

# P2 purinergic receptor modulation of cytokine production

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**Abstract** Cytokines serve important functions in controlling host immunity. Cells involved in the synthesis of these polypeptide mediators have evolved highly regulated processes to ensure that production is carefully balanced. In inflammatory and immune disorders, however, misregulation of the production and/or activity of cytokines is recognized as a major contributor to the disease process, and therapeutics that target individual cytokines are providing very effective treatment options in the clinic. Leukocytes are the principle producers of a number of key cytokines, and these cells also express numerous members of the purinergic P2 receptor family. Studies in several cellular systems have provided evidence that P2 receptor modulation can affect cytokine production, and mechanistic features of this regulation have emerged. This review highlights three separate examples corresponding to (1) P2Y<sub>6</sub> receptor mediated impact on interleukin (IL)-8 production, (2) P2Y<sub>11</sub> receptor-mediated effects on IL-12/23 output, and (3) P2X<sub>7</sub> receptor mediated IL-1 $\beta$  posttranslational processing. These examples demonstrate important roles of purinergic receptors in the modulation of cytokine production. Extension of these cellular observations to *in vivo* situations may lead to new therapeutic strategies for treating cytokine-mediated diseases.

**Key words** cytokines · dendritic cells · interleukin-1 · interleukin-8 · interleukin-12 · monocytes · nucleotides · purinergic receptors · tumor necrosis factor

## Abbreviations

|                  |                                       |
|------------------|---------------------------------------|
| LPS              | lipopolysaccharide                    |
| RA               | rheumatoid arthritis                  |
| IL               | interleukin                           |
| TNF              | tumor necrosis factor                 |
| IFN              | interferon                            |
| ERK              | extracellular signal regulated kinase |
| LDH              | lactate dehydrogenase                 |
| PGE <sub>2</sub> | prostaglandin E <sub>2</sub>          |
| GPCR             | G protein coupled receptor            |
| BZATP            | benzoylbenzoyl-ATP                    |

## Introduction

Cytokines comprise a heterogeneous group of polypeptides that mediate a variety of biological responses following their binding to specific receptors on target cells and tissues. Historically, cytokines (including lymphokines) were identified as leukocyte-derived soluble mediators that, when added to other leukocytes and/or to non-leukocyte targets, altered cellular behavior. Today, however, we realize that individual cytokines can be generated by cells of immune as well as non-immune origin, and that these polypeptides orchestrate a complex system of checks and balances controlling host immune and inflammatory processes. Our understanding of the importance that cytokines and cytokine signaling mechanisms serve in host defense mechanisms has been bolstered by the identification of genetic mutations within cytokine and/or cytokine receptor signaling complex genes that profoundly affect pathogen susceptibility [1, 2]. Likewise, genetically engineered mice that lack individual cytokines and/or cytokine receptors have provided a greater understanding of the capabilities

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possessed by these signaling molecules for promoting inflammatory and autoimmune states [3]. Moreover, the critical importance served by cytokines in mediating inflammation and autoimmunity has been underscored by the recent success of anti-cytokine biologics in the treatment of inflammatory diseases such as rheumatoid arthritis (RA) and Crohn's disease. For example, agents that neutralize tumor necrosis factor (TNF)  $\alpha$  (Enbrel, Remicade and Humira) have dramatically improved the treatment of RA [4–6]. In addition, the natural interleukin (IL)-1 receptor antagonist (Kineret) provides therapeutic benefit to RA patients and is reported to yield remarkable clinical outcomes when administered to patients suffering from a group of rare hereditary autoinflammatory disorders such as Muckle-Wells syndrome [7–9]. Ongoing clinical trials with agents that target IL-12 likewise are showing encouraging efficacy in the treatment of psoriasis and Crohn's disease [10, 11], and the list of anti-cytokine therapies entering clinical trials grows regularly [6]. Thus, cytokines and the signaling pathways engaged after they bind to receptors on cells represent attractive therapeutic targets for intervention of human autoimmune and inflammatory diseases. Studies over the past decade have suggested that purinergic receptor function, involving both P2Y and P2X family members, can modulate cytokine production and/or activity. This review focuses on several cellular systems where purinergic modulation of cytokine production has been demonstrated and mechanistic explanations have been sought. The cited studies signify that nucleotide receptor-mediated signaling can affect output of several important cytokines. In this context, pharmacological modulation of P2 receptors may represent a new therapeutic modality for treatment of cytokine-mediated disease processes in the future.

### Leukocyte expression of nucleotide receptors

Essentially all leukocyte populations express members of the P2 purinergic receptor superfamily; several recent reviews have detailed receptor expression patterns and the reader is directed to these for a more complete description [12, 13]. Human monocytes, for example, express mRNA encoding P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2X<sub>1</sub> and P2X<sub>7</sub> receptors [14–16]. Human lymphocytes similarly express mRNAs encoding P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub> receptors and members of the P2X family, including P2X<sub>7</sub>, are detected in both B and T-lymphocyte populations [15, 17–21]. Circulating human neutrophils express P2Y<sub>4</sub> and P2Y<sub>6</sub> mRNAs, and the P2X<sub>7</sub> receptor is reported to be present in these cells [22]. Human eosinophils and murine mast cells also are reported to contain the P2X<sub>7</sub> receptor [23, 24] as are rodent peritoneal macrophages [25, 26]; historically, many

ATP-dependent responses observed using murine macrophages were associated with activation of the P2Z receptor [27, 28]. Cloning and characterization of the P2X<sub>7</sub> receptor revealed that this polypeptide possessed functional attributes previously ascribed to the P2Z receptor [29, 30]. Thus, reference to the P2Z receptor was discontinued in favor of the P2X<sub>7</sub> receptor designation. Murine spleen macrophages, as well as the macrophage-like cell line J774, express multiple P2X and P2Y subtypes [31]. Murine and rat microglial cells, the brain's macrophage, also abundantly express P2X<sub>7</sub> receptors [32, 33].

A functional consequence often attendant to activation of the P2X<sub>7</sub> receptor is the opening of a nonselective pore that is permeable to large fluorescent molecules such as YoPro Yellow. Interestingly, the ability of monocytes and monocyte-like cells to demonstrate this pore activity is influenced by conditions under which these cells are cultured, suggesting that functional output of the P2X<sub>7</sub> receptor can be regulated [34–36]. Human monocytes demonstrate a change in receptor functionality without a corresponding change in the level of receptor mRNA expression, suggesting that receptor function can be regulated via posttranslational mechanisms [16].

Dendritic cells, which specialize in antigen presentation and are key producers of cytokines involved in maintenance of acquired immunity, also express members of the P2 receptor superfamily. Human monocyte-derived dendritic cells are reported to express mRNA for P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>5</sub>, P2Y<sub>6</sub>, P2Y<sub>10</sub>, P2Y<sub>11</sub>, and P2Y<sub>13</sub> from the P2Y receptor side of the family and P2X<sub>1</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, and P2X<sub>7</sub> from the P2X receptor lineage [37–39]. Murine dendritic cell lines also express the P2X<sub>7</sub> receptor [40].

### P2Y receptor-dependent regulation of cytokine production

*P2Y<sub>6</sub> receptor-mediated effects* The P2Y<sub>6</sub> receptor is selectively activated via UDP [41], and effects observed with this nucleotide often are attributed to activation of this receptor. Several studies have linked P2Y<sub>6</sub> function to IL-8 expression, a cytokine originally isolated as a neutrophil chemotactic factor and now designated as a member of the CXC family of chemokines [42]. IL-8 functions to promote influx of neutrophils to sites of injury and/or infection, and overproduction of IL-8 may contribute to several pathophysiological conditions including chronic lung inflammation and cancer [43]. When human monocytic THP-1 cells are treated with UDP, IL-8 output is enhanced and prior treatment of the cells with a P2Y<sub>6</sub> antisense oligonucleotide attenuates the magnitude of the cytokine response [44]. Likewise, P2Y<sub>6</sub> receptor transfected 1321N cells secrete greater levels of IL-8 in response to UDP challenge than do

their non-transfected counterparts [44]. Similarly, human promonocytic U937 cells stably transfected with human P2Y<sub>6</sub> receptor secrete IL-8 when stimulated with UDP [45]. P2Y<sub>6</sub> receptor-transfected U937 cells also generate TNF $\alpha$  and the chemokines MCP-1 and IP-10 in response to UDP activation. In contrast, UDP-challenged P2Y<sub>6</sub> receptor 1321N cell transfectants produce IL-8 but not other cytokine products [45]. The distinct response patterns displayed by the two transfected cell lines suggest that the cellular context in which the P2Y<sub>6</sub> receptor is placed impacts the pattern of cytokines/chemokines expressed. Interestingly, output of IL-8 from LPS stimulated THP-1 cells is decreased when apyrase is included in the cell culture medium [44], suggesting that nucleotides are released in response to LPS activation and these, in turn, activate purinergic receptors in an autocrine type of mechanism.

The mechanism by which the P2Y<sub>6</sub> receptor activates cytokine output has received limited investigation. The P2Y<sub>6</sub> receptor is a G<sub>q</sub>-coupled receptor [13, 46, 47] and thus is expected to promote activation of phospholipase C leading to the generation of inositol-1,4,5-triphosphate and elevation of intracellular Ca<sup>2+</sup> levels. In P2Y<sub>6</sub> receptor expressing THP-1 cells, the extracellular signal-regulated kinase (ERK) antagonist PD98059 impairs UDP-induced IL-8 output whereas the p38 stress kinase antagonist SB203580 is without effect. Thus, in this system the P2Y<sub>6</sub> receptor-induced rise in intracellular Ca<sup>2+</sup> appears to be coupled to an ERK-activated signaling cascade leading to IL-8 expression [44]. In osteoclasts, activation of the P2Y<sub>6</sub> receptor via UDP or the selective receptor agonist INS48823 induces a transient rise in intracellular Ca<sup>2+</sup> and activation of the transcription factor NF- $\kappa$ B [48]. Although cytokine output was not examined in the osteoclast system, activation of NF- $\kappa$ B often is associated with expression of cytokine genes [49] and oscillations in intracellular Ca<sup>2+</sup> concentrations have been linked to activation of this transcription factor [50].

With respect to inflammatory disease processes, no specific role for P2Y<sub>6</sub> receptors has been reported. However, tissue sections obtained from patients with inflammatory bowel disease are reported to contain T-cells within inflammatory lesions possessing enhanced expression of the P2Y<sub>6</sub> receptor [51]. As such, P2Y<sub>6</sub> receptor expression on T-cells may be upregulated at sites of inflammation and/or T-cells expressing this receptor may be preferentially recruited to these sites.

*P2Y<sub>11</sub> receptor-mediated effects impact dendritic cell function* The P2Y<sub>11</sub> receptor prefers ATP as its ligand, and the non-hydrolyzable ATP analog ATP $\gamma$ S often is employed *in vitro* as a surrogate ligand; the latter is not, however, a selective agonist of the P2Y<sub>11</sub> receptor [41, 47].

P2Y<sub>11</sub> receptor modulation of cytokine generation has been studied in some detail with human dendritic cells, with production of IL-12 being a major focus of these studies. IL-12 is composed of two distinct subunits, p40 and p35, which are covalently linked via an intermolecular disulfide bond to form the biologically active p70 species [52–54]. A related cytokine, IL-23, is composed of the same p40 subunit covalently bound to a unique p19 subunit [53, 54]. IL-12 and IL-23 are produced in abundance by activated antigen presenting cells such as monocytes and dendritic cells. When bound to target receptors on T-lymphocytes and natural killer (NK) cells, IL-12 activates interferon (IFN) $\gamma$  output, alters T-cell development, and affects NK cell killer activity [55]. IL-23 also activates T-cells and promotes IFN $\gamma$  output, but in this case the responding lymphocytes appear to represent a unique subpopulation of memory T-cells specializing in the production of the proinflammatory cytokine IL-17 [56, 57]. Together, IL-12 and IL-23 cooperate to shift the immune system toward a T helper (Th)1 state that is characteristic of inflammatory diseases such as RA and inflammatory bowel disease [58].

Application of ATP to human monocyte-derived dendritic cells (i.e., monocytes cultured for 6 days in the presence of granulocyte macrophage colony stimulating factor and IL-4) originally was reported to enhance expression of several cell surface molecules and to increase output of IL-12; this ATP effect was augmented by co-stimulation with TNF $\alpha$  [59]. Likewise, ATP but not UTP was reported to enhance expression of CXC chemokine receptor 4 by dendritic cells [60]. In these studies, the nature of the specific P2 receptor subtype(s) responsible for the dendritic cell cytokine response was not addressed.

While the above studies suggested that nucleotides may directly regulate cytokine output, more recent studies conducted with dendritic cells have focused on the role of extracellular nucleotides as modulators of cytokine output induced by other stimuli. For example, treatment of human monocyte-derived dendritic cells with either LPS or CD40 ligand promotes secretion of IL-1 $\alpha$ , IL-1 $\beta$ , TNF $\alpha$ , IL-6, and IL-12 (p70), and co-addition of ATP (250  $\mu$ M) along with the activation stimulus inhibits cytokine output [61]. In this same dendritic cell system, ATP does not inhibit output of IL-10 or IL-1 receptor antagonist, two cytokines possessing anti-inflammatory properties. The dendritic cell purinergic receptor responsible for the cytokine modulatory effects was not identified in this system, but the effect of ATP was mimicked by ADP but not by UTP.

In contrast to the simple pattern of cytokine inhibition noted above, other studies conducted with monocyte-derived dendritic cells suggest that the response elicited by extracellular nucleotides is complex in nature and dependent on the quantity of cytokine produced. For example, monocyte-derived dendritic cells treated with

TNF $\alpha$  or LPS generate greater quantities of IL-12 when simultaneously challenged with ATP (the ELISA kit employed in this study measured both IL-12p40 and IL-12p70). Comparison of the effectiveness of several ATP analogs suggests that the P2Y<sub>11</sub> receptor is responsible for enhancing cytokine expression [62]. In an extension of these findings, monocyte-derived macrophages were activated with a panel of different agonists (TNF $\alpha$ , LPS, or soluble CD40 ligand) in the absence or presence of ATP $\gamma$ S [63]. At agonist concentrations yielding low levels of IL-12p40 and TNF $\alpha$  output, ATP $\gamma$ S (200  $\mu$ M) increases secreted levels of these two polypeptides. However, at agonist concentrations yielding higher levels of IL-12p40 and TNF $\alpha$  output, ATP $\gamma$ S inhibits their output. Notably, LPS (but not TNF $\alpha$  or CD40) stimulates secretion of the bioactive, heterodimeric form of IL-12 (i.e., IL-12p70) and ATP $\gamma$ S antagonizes IL-12p70 output at all tested LPS concentrations. It is known from other studies that the p40 and p35 subunits of IL-12 can be regulated independently [64]; lack of coordinated synthesis may help to explain why ATP $\gamma$ S can enhance IL-12p40 but inhibit IL-12p70 output in response to LPS challenge.

The ATP response observed in the dendritic cell system assumes an even greater complexity when the output of IL-12 and IL-23 are compared. Human monocyte-derived dendritic cells activated with intact *E. coli* produce both IL-12 and IL-23. In response to this challenge, IL-12p40, IL-12p35, and IL-23p19 message levels increase and levels of IL-12 and IL-23 released extracellularly increase accordingly [65]. Addition of ATP (250  $\mu$ M) to the medium during bacterial challenge decreases IL-12p40 and IL-12p35 message levels but increases IL-23 message levels. Likewise, whereas *E. coli*-induced secretion of IL-12p70 and IL-12p40 from dendritic cells is inhibited by ATP, secretion of bioactive IL-23 is enhanced. Since both IL-12 and IL-23 share the IL-12p40 subunit, the opposite effects achieved by ATP with respect to output of these two cytokines is somewhat surprising. However, this may reflect that synthesis of the p40 subunit can exceed that of the p19 subunit and secretion of the bioactive IL-23 heterodimer requires simultaneous expression of both subunits [66]. ATP $\gamma$ S and AR-C67085 (a synthetic nucleotide analog) are more potent than ATP at inhibiting *E. coli*-induced output of IL-12p70 and IL-12p40. In contrast, these two nucleotides are ineffective as enhancers of IL-23; ADP, however, is as effective as ATP at enhancing *E. coli*-induced IL-23 output. On this basis, it was concluded that the P2Y<sub>11</sub> receptor is responsible for the nucleotide-mediated antagonism of IL-12 production whereas a separate, ADP-sensitive P2 receptor subtype is responsible for promoting IL-23 production [65].

Sorting out the identity of P2 receptors that mediate the aforementioned effects of nucleotides on dendritic cell

cytokine output is complicated by the existence of cell surface nucleotidases such as CD39 [37]. These enzymes can act on exogenously added nucleotides to generate metabolites that may possess altered selectivity for P2 receptors relative to the parent nucleotide originally added to the culture medium. In this light, it is interesting to note that ADP and several related nucleotides also are reported to inhibit IL-12p70, IL-12p40, and TNF $\alpha$  output from LPS-activated dendritic cells [67]. Based on the inability of ADP to act as an agonist of the P2Y<sub>11</sub> receptor and the nature of the signaling response induced by this nucleotide, the cytokine modulatory effects induced by ADP in this system may reflect activation of a novel dendritic cell P2 receptor subtype [67].

Mechanistic features attendant to P2Y<sub>11</sub> receptor activation have been investigated and changes to intracellular cAMP concentrations appear important to the cytokine modulatory response. The P2Y<sub>11</sub> receptor employs both Gq- and Gs-type G-proteins in mediating signaling [47]; the P2Y<sub>11</sub> receptor is unique amongst P2 receptors in its ability to employ Gs and, in turn, to activate adenylate cyclase. Activation of adenylate cyclase and the associated rise in cAMP levels appear to be responsible for both the ATP-induced rise in IL-23 and decline in IL-12 from *E. coli*-induced human dendritic cells. This conclusion is based on several observations. First, substitution of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) for ATP in the dendritic cell system produces a similar outcome with respect to inhibition of IL-12 and augmentation of IL-23 output [63, 65]. PGE<sub>2</sub> signals via G-protein-coupled prostaglandin receptors, and two of these receptors, EP<sub>2</sub> and EP<sub>4</sub>, transmit signals via Gs [68]. Studies conducted in genetically altered mice support the involvement of the EP<sub>4</sub> receptor in mediating the inhibitory effect on IL-12 production; LPS-activated bone marrow-derived macrophages derived from wild-type mice but not from EP<sub>4</sub> receptor-deficient animals produce less IL-12 in the presence of PGE<sub>2</sub> [69]. Second, activation of other G protein coupled receptors (GPCRs) that engage adenylate cyclase, such as histamine (H<sub>2</sub>) and adenosine A<sub>2a</sub> receptors, inhibit IL-12 output [70]. Third, the ability of ATP analogs to alter IL-12 output correlates with their impact on intracellular cAMP levels [62]. Finally, treatment of *E. coli*-activated dendritic cells with forskolin, an agent that activates adenylate cyclase independently of GPCR activation, also inhibits IL-12 (protein) output while enhancing IL-23p19 (message) expression [65]. In view of the ability of the aforementioned agents to elevate cAMP and to produce reciprocal effects on IL-12 and IL-23 output from dendritic cells, it is surprising that ATP $\gamma$ S and AR-C67085, two effective agonists of the P2Y<sub>11</sub> receptor, cause inhibition of IL-12 output without enhancing IL-23 expression [65]. Notably, ADP is able to enhance IL-23p19 message expression by *E. coli*-activated dendritic cells [65].



The reciprocal effects observed with respect to production of IL-12 and IL-23 in the presence of ATP, therefore, may reflect activation of the P2Y<sub>11</sub> receptor by ATP and, as a result of hydrolysis of the added ATP via ecto-nucleotidases, simultaneous activation of a novel type of P2 receptor that is activated via ADP and coupled to Gs.

Although dendritic cell cytokine output is well documented to be subject to regulation via nucleotides *in vitro*, there is little information to suggest that this type of regulation takes place *in vivo*. A recent report noted, however, that intradermal injection of ATP $\gamma$ S into mice results in an enhanced contact hypersensitivity response, and this effect may be achieved as a result of activation of Langerhans cells, a subclass of dendritic cells [71]. Murine Langerhans cells, like other dendritic cells, express the ecto-nucleotidase CD39, and CD39-deficient mice display an exacerbated skin inflammatory response when irritant chemicals such as croton oil are applied topically [72].

### P2X receptor-dependent regulation of cytokine production

With the exception of the P2X<sub>7</sub> receptor, functional responses attendant to activation of P2X receptor family members are not generally associated with cytokine modulation. Members of this family certainly can impact inflammatory processes as evidenced by the attenuated pain responses observed following administration of selective P2X<sub>3</sub> receptor antagonists to rodents [73]. These effects, however, are not directly linked to cytokine output but, rather, to effects associated with modulation of sensory afferent neurons. Therefore, a discussion of how P2X receptors affect cytokine output is limited in scope to the role of the P2X<sub>7</sub> receptor. Although selective agonists and/or antagonists of the P2X<sub>7</sub> receptor have to this point not been available, the P2X<sub>7</sub> receptor possesses atypical features that often allow its function to be implicated during *in vitro* studies. Most notable amongst these features is the requirement that high ATP concentrations, often in excess of 500  $\mu$ M, be employed to achieve receptor activation when cells are maintained in physiological media. Benzoylbenzoyl-ATP (BZATP) often is employed as an agonist of the P2X<sub>7</sub> receptor. Although this agent is reported to be a more effective agonist than ATP [74], it is not specific for the P2X<sub>7</sub> receptor [75]. Likewise, oxidized ATP often is employed as an antagonist of the P2X<sub>7</sub> receptor [76], but this agent also acts in a P2 receptor-independent manner [77].

**P2X<sub>7</sub> receptor-mediated effects** As noted earlier, the P2X<sub>7</sub> receptor is present on a number of leukocyte populations

including monocytes and tissue macrophages. When challenged appropriately, cells of monocyte/macrophage lineage are abundant producers of proinflammatory cytokines including IL-1 and TNF $\alpha$ . With respect to TNF $\alpha$  output, both enhancing and inhibitory responses have been associated with P2X<sub>7</sub> receptor activation. Cultured rat microglia treated with 1 mM ATP, for example, increase expression of TNF $\alpha$  mRNA and secrete this proinflammatory cytokine [78]. In this cellular system, the TNF $\alpha$ -enhancing effect of ATP is dependent on an influx of extracellular calcium, and is suppressed by inhibitors of ERK (PD098059) and p38 (SB203580) mitogen activated kinases. Likewise, ATP is reported to enhance TNF $\alpha$  output from murine RAW 264.7 macrophages both in the absence and presence of LPS [79]; the purinergic receptor responsible for this effect in RAW cells is unknown. On the other hand, ATP is reported to inhibit TNF $\alpha$  release from LPS-activated murine peritoneal macrophages [80]; this antagonism does not appear to result from activation of the P2X<sub>7</sub> receptor as UTP and UDP mimic the action of ATP but are not P2X<sub>7</sub> receptor agonists. Similarly, ATP inhibits LPS-induced release of TNF $\alpha$  from rat cortical astrocytes [81]. In this cellular system mM concentrations of ATP are required for the biological response, and the effect of ATP is mimicked by BZATP, properties consistent with involvement of the P2X<sub>7</sub> receptor. Thus, the net effect observed with respect to ATP's ability to modulate TNF $\alpha$  output is dependent on the cellular context.

The most extensively studied cytokine modulatory role involving the P2X<sub>7</sub> receptor relates to its ability to promote IL-1 $\beta$  posttranslational processing. IL-1 $\beta$  is a multifaceted proinflammatory cytokine produced predominantly by cells of the monocyte/macrophage lineage [82]. Resting monocytes and macrophages do not constitutively produce IL-1 $\beta$ , but following challenge with an activating stimulus such as LPS, these cells rapidly engage in the production of large quantities of proIL-1 $\beta$ . This precursor polypeptide (31 to 35 kDa in mass) must be proteolytically processed by caspase-1 to generate the mature biologically active 17 kDa cytokine species [83–85]. Importantly, proIL-1 $\beta$  lacks a leader sequence and, as a result, the newly synthesized polypeptides accumulate intracellularly within the cytosol of LPS-activated cells. In contrast to IL-1 $\beta$ , caspase-1 is constitutively expressed by monocytes and macrophages; this cysteine protease also resides in the cytoplasm as an inactive zymogen [86]. In the absence of additional stimulation, only a very low percentage of the newly synthesized proIL-1 $\beta$  polypeptides produced by LPS-treated cells is processed by caspase-1 and released extracellularly [87]. However, in the presence of an appropriate activation stimulus, procaspase-1 is converted to its catalytically active form, proIL-1 $\beta$  subsequently is cleaved to its mature 17 kDa species, and the mature

cytokine is released extracellularly where it can engage receptors on target cells; a number of agents have been reported to facilitate this posttranslational processing *in vitro* including various toxins [88–92], defensin-like peptides [93, 94], and  $K^+$  ionophores [95–97].

David Chaplin and his group first demonstrated that ATP can act as a trigger to promote IL-1 $\beta$  posttranslational processing from LPS-activated murine peritoneal macrophages [98]. When incubated in the absence of ATP, LPS-activated macrophages labeled with [ $^{35}$ S]methionine were shown to possess abundant quantities of the 35 kDa proIL-1 $\beta$  polypeptide, but radiolabeled cytokine products were not released extracellularly. Following addition of ATP to the medium, however, the LPS-activated/[ $^{35}$ S]methionine-labeled cells released IL-1 $\beta$  to the medium and the majority of the externalized cytokine was efficiently converted to the mature 17 kDa species. The ATP-treated macrophages also released the cytoplasmic enzyme lactate dehydrogenase (LDH) and possessed a DNA fragmentation profile that was characteristic of an apoptotic cellular response. Although the identity of the receptor responsible for mediating these effects was not addressed, the high concentration of ATP employed (5 mM) suggested P2X $_7$  receptor involvement. The ability of ATP to promote IL-1 $\beta$  posttranslational processing by murine peritoneal macrophages is not limited to *in vitro* cultures. Mice primed with LPS *in vivo* contain peritoneal macrophages that are laden with proIL-1 $\beta$ , but lavage of the peritoneal cavities of these mice yields minimal quantities of the mature cytokine species. Following a subsequent intraperitoneal injection of ATP into the LPS-primed animals, however, large quantities of mature IL-1 $\beta$  are recovered in the lavage fluids [99]. Thus, ATP acts as an effective agonist of IL-1 $\beta$  posttranslational processing both *in vitro* and *in vivo*.

Subsequent studies demonstrated that ATP is an effective stimulus for promoting IL-1 $\beta$  posttranslational processing by a number of different cell types including human monocytes/macrophages [100–102] and human and mouse microglial cells [103–105]. In all cases, the cytokine response requires concentrations of ATP >1 mM, a requirement consistent with activation of the P2X $_7$  receptor. Involvement of the P2X $_7$  receptor is further supported by the observation that KN62 inhibits ATP-induced IL-1 $\beta$  posttranslational processing by LPS-activated human monocytes [102]; this agent is a potent inhibitor of P2X $_7$  receptor-mediated functions [106, 107]. Furthermore, an antibody generated against the P2X $_7$  receptor blocks ATP-induced IL-1 $\beta$  release from LPS-activated human monocytes [108]. Analysis of peritoneal macrophages obtained from mice genetically engineered to lack the P2X $_7$  receptor provided the final piece of evidence that P2X $_7$  receptor function is necessary for ATP-induced IL-1 $\beta$  posttranslational processing [26]. Macrophages obtained from both

wild type and P2X $_7$ -deficient mice generate equivalent quantities of newly synthesized proIL-1 $\beta$  in response to LPS challenge. However, in the absence of a secondary stimulus neither macrophage population releases mature IL-1 $\beta$  to the medium. Following treatment with 5 mM ATP, wild type but not P2X $_7$  receptor-deficient macrophages externalize large quantities of mature IL-1 $\beta$ . In contrast, both macrophage populations release mature IL-1 $\beta$  when treated with the potassium ionophore nigericin, indicating that the P2X $_7$  receptor-deficient macrophages are competent to process proIL-1 $\beta$  but absence of the P2X $_7$  receptor prevents them from doing so when challenged with ATP [26].

*Signaling pathways associated with P2X $_7$  receptor activation* Mechanistic elements engaged as a result of P2X $_7$  receptor activation that are responsible for initiating IL-1 $\beta$  posttranslational processing are not completely understood, but studies to date have provided insight into this atypical secretory process. ATP acting via the P2X $_7$  receptor activates a number of intracellular kinases including members of the MAP kinase family ERK and JNK [23, 109–112], Rho effector kinases [113, 114], and the protein tyrosine kinase p56<sup>lck</sup> [115]. Correspondingly, increased phosphorylation of various intracellular polypeptides including the receptor itself are observed post-ATP activation [116–118]. P2X $_7$  receptor operation also is associated with activation of various transcription factors [119, 120], enhanced production of reactive oxygen species [121], mitochondrial membrane depolarization [122], and activation of phospholipase D [123]. It remains to be established whether any of these changes are required for IL-1 $\beta$  posttranslational processing, although a  $Ca^{2+}$ -independent phospholipase inhibitor (bromo-enol lactone) and a tyrosine kinase inhibitor (AG-126) are reported to inhibit ATP-induced IL-1 $\beta$  output [124, 125]. As a ligand-gated ion channel, the P2X $_7$  receptor also promotes rapid changes in ionic homeostasis following its activation [126]. Prolonged ligation of the receptor can result in complete membrane depolarization, a process that is likely to involve opening of the P2X $_7$  receptor operated pore [126]. In several cellular systems, a rise in intracellular  $Ca^{2+}$  promoted by the P2X $_7$  receptor is necessary for IL-1 $\beta$  posttranslational processing [127, 128]. Similarly,  $K^+$  efflux mediated via P2X $_7$  receptor activation in LPS-stimulated monocytes and macrophages is necessary for efficient IL-1 $\beta$  posttranslational processing [96, 97, 100, 101, 125, 127]. Simply increasing the medium  $K^+$  ion concentration can completely inhibit ATP-induced IL-1 $\beta$  output [96, 100, 101, 129]; as such,  $K^+$  efflux appears to serve a key role in the cellular process.

How these changes to intracellular  $K^+$  and  $Ca^{2+}$  ion levels regulate IL-1 $\beta$  posttranslational processing remains to be established. In the case of  $K^+$ , the P2X $_7$  receptor-induced changes may facilitate activation of caspase-1. As

noted earlier, procaspase-1 is expressed constitutively by monocytes and macrophages and resides in the cytoplasmic compartment as a latent zymogen. Ligation of the P2X<sub>7</sub> receptor leads to rapid activation of caspase-1 and, like mature IL-1 $\beta$ , the activated protease is released to the medium [130]. Recent studies have indicated that activation of procaspase-1 requires assembly of a large protein scaffold termed the inflammasome [131, 132]. Components of the inflammasome may include a NALP (NALP1, 2 or 3), the speck like protein ASC, and procaspase-5 [132–135]. The importance of ASC in ATP-induced IL-1 $\beta$  posttranslational processing is highlighted by the demonstration that LPS-activated peritoneal macrophages isolated from ASC-deficient mice fail to generate mature IL-1 $\beta$  in response to ATP challenge [136]. In a resting cell, the NALP polypeptide appears to exist in an inactive monomeric conformation, but this polypeptide may undergo a conformational change in response to an appropriate effector leading to its association with ASC and procaspases [135]. This association involves several protein-protein interaction domains including PYRIN and caspase recruitment domains (CARDs). The resulting protein ensemble positions the procaspases in close proximity leading to their proteolytic activation [135]. Studies of inflammasome assembly using broken cell preparations have demonstrated that extracts prepared from cells briefly treated with mM concentrations of ATP are more effective at generating mature caspase-1 and mature IL-1 $\beta$  than are comparable extracts prepared from non-ATP treated cells. Moreover, when the ATP treatment is performed in the presence of high extracellular K<sup>+</sup> (thus limiting K<sup>+</sup> efflux from the ATP-treated cells), the resultant inflammasome activity in the cell-free extract is reduced [125]. These findings, therefore, suggest that K<sup>+</sup> efflux may facilitate inflammasome assembly and/or its activation.

As noted above, changes in intracellular Ca<sup>2+</sup> levels also contribute to IL-1 $\beta$  posttranslational processing. ATP-induced changes in intracellular Ca<sup>2+</sup> may result from Ca<sup>2+</sup> entry via P2X receptors as well as from Ca<sup>2+</sup> release from intracellular stores mediated via P2Y receptors. In LPS-activated mouse peritoneal macrophages, Ca<sup>2+</sup> release from intracellular stores is required for ATP-induced IL-1 $\beta$  maturation and release, but in this system the cytokine response does not require Ca<sup>2+</sup> influx from the medium [127]. Likewise, ATP-induced IL-1 $\beta$  output from LPS-activated human monocytes is unaffected by removal of extracellular Ca<sup>2+</sup> [129]. However, LPS-activated human THP-1 cells [137] and HEK293 engineered to express both the P2X<sub>7</sub> receptor and mature IL-1 $\beta$  [128] require influx of extracellular Ca<sup>2+</sup> for optimal cytokine output. Therefore, the nature of the Ca<sup>2+</sup> requirement is dependent on the type of cell being analyzed. Interestingly, a recent study concluded that opening of the P2X<sub>7</sub> receptor-activated pore is depen-

dent on Ca<sup>2+</sup> [138]. Although a role for the pore in IL-1 $\beta$  posttranslational processing has not been established, perhaps the Ca<sup>2+</sup> requirement for IL-1 maturation and release relates to this activity. Moreover, Ca<sup>2+</sup> influx via the P2X<sub>7</sub> receptor recently was linked to a pseudoapoptotic state characterized by phosphatidylserine movements within the plasma membrane and to cytoskeletal disruption and zeiotic membrane distortions [139].

*Externalization of mature IL-1 $\beta$  following P2X<sub>7</sub> receptor activation* A number of different mechanisms have been proposed to explain how mature IL-1 $\beta$  is released to the extracellular environment following ATP stimulation. LPS-activated murine peritoneal macrophages and human monocytes exposed to mM concentrations of ATP *in vitro* release mature IL-1 $\beta$  via a process that is accompanied by release of the cytoplasmic marker enzyme LDH [98, 140]. Although the kinetics of appearance of LDH within the medium of ATP-treated mouse peritoneal macrophages lags behind the appearance of mature IL-1 $\beta$  [140], release of the former to the medium suggests that plasma membrane latency is lost during the ATP-induced process. Thus, one mechanism proposed for the release of mature IL-1 $\beta$  is that the producing cell dies, possibly via an apoptotic-like process, and intracellular components are released passively from the cell following disruption of the plasma membrane [98]. On the other hand, evidence exists to suggest that release of mature IL-1 $\beta$  is a facilitated process. For example, release of mature IL-1 $\beta$  from LPS-activated murine macrophages in response to ATP challenge is blocked by non-selective agents that are known to antagonize transport function of ABC1, a membrane-bound protein that functions in cholesterol transport [141]. On this basis, ABC1 was proposed to facilitate transport of mature IL-1 $\beta$  [141]. Moreover, when LPS-activated THP-1 cells are treated with 300  $\mu$ M BZATP, microvesicles which contain bioactive IL-1 $\beta$  are rapidly shed from the cell surface [137]. A similar process has been reported to occur in mixed cultures of rat primary astrocytes and microglia [142]. In this case, ATP released from astrocytes appears to promote shedding of microvesicles from neighboring microglia containing IL-1 $\beta$ . Details of how the shed microvesicles subsequently release their content of cytokine have yet to be defined. An even more elaborate system for externalization of mature IL-1 $\beta$  has been proposed based on immunohistochemical observations that co-localized proIL-1 $\beta$  and lysosomal enzymes within cytoplasmic vesicles [143, 144]. In response to ATP, these vesicles appear to fuse with the plasma membrane via a process that is triggered by K<sup>+</sup> depletion and dependent on phospholipase activation [144]. To what extent each of these different mechanisms contributes to the release of IL-1 $\beta$  *in vivo* remains to be established.

ATP's ability to promote IL-1 $\beta$  posttranslational processing *in vitro* via the P2X<sub>7</sub> receptor is well documented, but evidence demonstrating that the P2X<sub>7</sub> receptor functions in this capacity *in vivo* is limited. Moreover, no evidence has thus far been presented to suggest that P2X<sub>7</sub> receptor levels and/or activity are altered in human inflammatory diseases. A number of single nucleotide polymorphisms have been identified in the human P2X<sub>7</sub> receptor gene [145–151] and these can lead to impaired ATP-induced IL-1 $\beta$  (and IL-18) posttranslational processing *in vitro* [152, 153]. Thus, it will be of great interest to determine whether individuals possessing these functionally impaired P2X<sub>7</sub> receptor phenotypes are less susceptible to inflammatory disorders. Two independent P2X<sub>7</sub> receptor-deficient mouse lines have been generated [26, 154]. Although these receptor-deficient mice are overtly normal, when subjected to various challenges they display attenuated inflammatory responses. For example, after treatment with a panel of anticollagen antibodies to induce an RA-like disease state, joints recovered from wild type mice display a more pronounced inflammatory cell infiltrate and greater cartilage destruction than do joints recovered from P2X<sub>7</sub> receptor-deficient mice [26]. In similar murine models of arthritis, administration of neutralizing anti-IL-1 antibodies are known to suppress the inflammatory response [155–157]. Therefore, the protection afforded by deletion of the P2X<sub>7</sub> receptor is consistent with the knockout mice possessing a diminished capacity to generate mature IL-1 $\beta$ . Indeed, following intraplantar injection of Freund's complete adjuvant, extracts of the injected paws obtained from wild type mice contain greater levels of IL-1 $\beta$  than do comparable extracts obtained from P2X<sub>7</sub> receptor-deficient mice [154]. Moreover, P2X<sub>7</sub> receptor-deficient mice display less hypersensitivity to the adjuvant challenge than do their wild type counterparts. These *in vivo* disease model studies, therefore, suggest that antagonism of the P2X<sub>7</sub> receptor may offer a novel therapeutic approach for the treatment of inflammatory disorders. As several pharmaceutical companies appear to be engaged in a search for antagonists of the P2X<sub>7</sub> receptor [158–160], the ability to selectively modulate this receptor pharmacologically in animal models and man may soon be possible.

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